EXHIBIT A

The Journal of Immunology

HSP110-HER2/*neu* Chaperone Complex Vaccine Induces Protective Immunity Against Spontaneous Mammary Tumors in HER-2/*neu* Transgenic Mice[1]

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Heat shock proteins (HSPs) are shown to be strong immunoadjuvants, eliciting both innate and adaptive immune responses against cancers. HSP110 is related in sequence to HSP70 and is ~4-fold more efficient in binding to and stabilizing denatured protein substrates compared with HSP70. In the present study we evaluated the ability of a heat shock complex of HSP110 with the intracellular domain (ICD) of human HER-2/*neu* to elicit effective antitumor immune responses and to inhibit spontaneous mammary tumors in FVB-*neu* (FVBN202) transgenic mice. The HSP110-ICD complex was capable of breaking tolerance against the rat neu protein and inhibiting spontaneous mammary tumor development. This vaccine induced ICD-specific IFN-γ and IL-4 production. Depletion studies revealed that $CD8^+$ T cells were involved in protection against challenge with mouse mammary tumors, whereas $CD4^+$ T cells revealed partial protection. Increased IgG2a Ab titer in the sera of tumor-free animals after vaccination and elevated $CD4^+$ $CD25^+$ regulatory T cells in the PBL of tumor-bearing animals suggested that IFN-γ-producing Th1 cells may be responsible for partial protection of $CD4^+$ T cells against the mammary tumor challenge, whereas $CD4^+CD25^+$ regulatory T cells (Th2 cells) may suppress the antitumor immune responses. Together, these results suggest that HSP110-ICD complex can elicit effective IFN-γ-producing T cells against spontaneous mammary tumors and that up-regulation of $CD4^+$ $CD25^+$ regulatory T cells may prevent complete eradication of the tumor following immunotherapy. *The Journal of Immunology*, 2003, 171: 4054–4061.

Certain heat shock proteins (HSPs)[3] have been shown to act as effective vaccines against a cancer when they are purified from the same cancer (1–5). This approach takes advantage of the peptide-binding properties of these stress proteins, which is also responsible for their functions as molecular chaperones in numerous processes (6, 7). Immunoadjuvant properties of HSPs stem from their ability 1) to induce APCs to secrete proinflammatory cytokines, such as IL-1β, IL-6, IL-12, and TNF-α, that induce innate immunity (7–10); 2) to shuttle the associated Ag to both MHC class I and MHC class II Ag presentation pathways that provide the immune system with signal I (11, 12); and 3) to up-regulate costimulatory molecules on APCs that provide the immune system with signal II (12–14). Therefore, HSPs interact with APCs via specific receptors and act as a double-edge sword, stimulating both innate and adaptive immune responses.

HSP110 is a large stress protein that is distantly related in sequence to the HSP70 family. It has been shown to be ~4-fold more efficient in binding to and stabilizing denatured protein substrates compared with HSP70 (5, 15). This strong chaperoning property of HSP110 allows it to not only bind reporter proteins useful in chaperoning studies, but also to bind important tumor protein Ags, thereby defining a new approach to heat shock vaccine formulation.

We have recently examined this HSP-based vaccine strategy using the chaperoning function of HSP110 to generate a natural noncovalent complex with the intracellular domain (ICD) of HER-2/*neu* (16). We showed that HSP110 formed a noncovalent binding complex with ICD at an ~1/1 molar ratio (16). This vaccine was capable of eliciting an ICD-specific immune response in A2/Kb transgenic mice. Both $CD4^+$ and $CD8^+$ T cells were involved in the ICD-specific IFN-γ production, as determined by ELISPOT assay, following depletion of $CD4^+$ and/or $CD8^+$ T cells in vivo (16). We have made similar findings using melanoma gp100 in a noncovalent complex with HSP110. This HSP110-gp100 complex elicited antitumor immune responses against the gp100-overexpressing B16 melanoma cells (5).

HER-2/*neu* was selected as an Ag of choice, since it is a well-known breast tumor-associated Ag that has been targeted for immunotherapy of human breast and ovarian cancers (17–20). Evaluation of the antitumor potential of the HSP110-ICD vaccine approach requires an animal model that resembles human breast cancer in vivo. FVBN202 transgenic mice are transgenic for the rat *neu* oncogene and thus are under severe genetic pressure to develop spontaneous breast tumors (21, 22). By the time animals are 6–10 mo of age (corresponding to human middle age), 100% of animals develop mammary tumors. The development and histology of these mammary tumors has a striking resemblance to those seen in human breast cancer. Most human breast cancer patients have

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[3] Abbreviations used in this paper: HSP, heat shock protein; ANV, Ag-negative variant; h, human; HER-2/neu, human epidermal growth factor receptor-2/neu; ICD, intracellular domain; MFI, mean fluorescence intensity; MMTC, mouse mammary tumor cells; MMTV, mouse mammary tumor virus; r, rat.

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pre-existing immunity against HER-2/*neu*, whereas FVBN202 transgenic mice do not have such pre-existing immunity. Thus, breaking tolerance against the neu protein is necessary to generate antitumor immunity against the tumor (23, 24).

To evaluate the ability of HSP110-ICD complex to inhibit spontaneous mammary tumor, we used human (h) ICD, which is 92% homologous to rat (r) ICD. It has been shown previously that human HER-2/*neu* can break tolerance against rat neu protein (25). We investigate here the ability of the HSP110-ICD complex to break tolerance against the neu protein and to inhibit spontaneous mammary tumors in the FVBN202 transgenic mice. We also investigate the mechanisms for the development of mammary tumors and the failure of immunotherapy to completely eradicate the tumor. We found that induced IFN-γ-secreting T cells were involved in the antitumor protection. Since it was previously reported that breast cancer patients have elevated $CD4^+$ $CD25^+$ regulatory T cells in their peripheral blood compared with healthy individuals (26), we investigated whether up-regulation of these regulatory/suppressor T cells may favor tumor development and prevent complete eradication of mammary tumors in FVBN202 transgenic mice. We found that the development of spontaneous mammary tumor coincided with the up-regulation of $CD4^+$ $CD25^+$ T cells.

Materials and Methods

Animals

FVBN202 is a rat neu transgenic mouse model in which 100% of females develop spontaneous mammary tumors by 8–10 mo of age, with many features similar to human breast cancer. These mice overexpress an unactivated rat *neu* transgene under the regulation of mouse mammary tumor virus (MMTV) promoter (21). The FVBN202 transgenic mice ($H\text{-}2^q$) were provided by Dr. A. Sood (Roswell Park Cancer Institute, Buffalo, NY), and females were used in the present study.

Recombinant proteins

Mouse recombinant HSP110 was made using the pBacPAK.His vector (Clontech Laboratories, Palo Alto, CA). This vector-carrying HSP110 gene is cotransfected with BacPAK6 viral DNA into Sf21 insect cells using a BacPAKTM Baculovirus Expression System Kit (Clontech Laboratories), followed by amplification of the recombinant virus and purification of HSP110 protein using nickel-chelated-nitrilotriacetic acid-agarose by using a protein assay kit (Bio-Rad, Hercules, CA). Human and rat recombinant ICD were provided by Dr. R. Henderson (Corixa Corp., Seattle, WA). Endotoxin levels in recombinant proteins (~30–50 endotoxin units/mg protein) were measured using a *Limulus* amebocyte lysate kit (BioWhittaker, Walkersville, MD).

Preparation of HSP110-ICD complex

The HSP110-ICD complex was generated as previously described (16). Briefly, HSP110 was incubated with ICD in a 1/1 molar ratio at 43°C for 30 min and then at 37°C for 1 h. Binding was evaluated by immunoprecipitation using rabbit anti-mouse HSP110 antiserum (1/200) and protein A/Sepharose CL-4B (20 μl/ml; Amersham Pharmacia Biotech, Uppsala, Sweden). Samples were then subjected to SDS-PAGE (10%), followed by either Gel-Blue staining or probing with mouse anti-human ICD antiserum (1/10,000; Corixa Corp.) in a Western blot analysis using HRP-conjugated sheep anti-mouse IgG (1/5,000; Amersham Pharmacia Biotech, Piscataway, NJ). The results indicated that HSP110 chaperones ICD in an ~1/1 molar ratio (16).

Tumor cell lines

Mouse mammary tumor cells (MMTC) overexpressing the neu protein and the neu Ag-negative variant (ANV) of MMTC with low expression of the neu protein were supplied by Dr. K. Knutson (University of Washington, Seattle, WA). MMTC was derived from neu-overexpressing spontaneous mammary tumors developed in FVBN202 transgenic mice. ANV was prepared by growing MMTC in parental FVB mice in which neu expression was suppressed under immune pressure, and clones with normal neu expression were selected and expanded. Both cell lines were cultured in RPMI 1640 supplemented with 10% FBS. Plates that were uniformly cubical epithelial cells with overexpression of neu (MMTC) or low expression of neu (ANV) were frozen, then thawed as needed. Staining for neu protein expression was conducted periodically, using c-neu Ab (Ab-4; Oncogene Research Products, San Diego, CA) in a flow cytometry-based analysis.

ELISPOT

The ICD-specific T cell responses by the immunized animals were evaluated using an ELISPOT assay as described previously (16). Briefly, 96-well filtration plates (Millipore, Bedford, MA) were coated with 10 μg/ml of rat anti-mouse IFN-γ (BD PharMingen, San Diego, CA) or rat anti-mouse IL-4 Ab (eBioscience, San Diego, CA) and subsequently blocked with RPMI 1640 medium containing 10% FBS. RBC were lysed by Tris-NH_4Cl, and 50 μl of the splenocytes (5×10^5 cells/well) were added to the wells and incubated with 50 μl of Con A (5 μg/ml), rICD (10 μg/ml), hICD (20 μg/ml), or HSP110 (20 μg/ml) in complete medium (10% FBS, penicillin/streptomycin (50 U/ml), L-glutamine (2 mM), and 2-ME (1 mM)) at 37°C in an atmosphere of 5% CO_2 for 20–24 h. The plates were then washed extensively and incubated with 5 μg/ml biotinylated anti-mouse IFN-γ (BD PharMingen) or biotinylated anti-mouse IL-4 Ab (eBioscience), followed by pulsing with 0.2 U/ml alkaline phosphatase avidin D (Vector Laboratories, Burlingame, CA). Positive spots were developed by adding 50 μl/well 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium solution (Roche, Indianapolis, IN) and were counted using a Vision ELISPOT reader (Carl Zeiss, New York, NY).

Depletion of T cell subsets

In vivo depletion of T cell subsets was conducted as described previously (27). The GK1.5 (anti-CD4) and 2.43 (anti-CD8) hybridomas were provided by Dr. D. Pardoll (The Johns Hopkins University, Baltimore, MD), and the Abs were purified by ammonium sulfate precipitation from ascites of SCID mice injected i.p. with the hybridoma cells. The depletions were started 4 days before the tumor challenge. Each animal was injected i.p. with 250 μg of the mAbs on 3 subsequent days before and twice a week after the challenge. Animals were depleted of $CD4^+$, $CD8^+$, or $CD4^+/CD8^+$ T cells. Depletion of the lymphocyte subsets was assessed on the day of challenge and weekly thereafter by FACS analysis of splenocytes stained with mAbs GK1.5 or 2.43, followed by FITC-conjugated rat anti-mouse IgG (BD PharMingen). For each time point analysis, >98% of the appropriate subset was achieved. The percentage of $CD4^+$ T cells did not change after $CD8^+$ T cell depletion, and percentage of $CD8^+$ T cells also did not change after $CD4^+$ T cell depletion.

Tumor growth

Tumor volume (V) was calculated by: $V = L \times W2/2$ for each tumor, where L is tumor length, and W is tumor width, and total tumor burden was calculated by the sum of the tumor volumes in each mouse. Mice were sacrificed before a tumor mass exceeded 2 cm in diameter.

ELISA

IgG1 and IgG2a Ab responses were measured in the sera of tumor-free and tumor-bearing animals using ELISA. Briefly, 96-well ELISA plates were coated with rICD (20 μg/ml) or HSP110 (20 μg/ml), then blocked with 2% skim milk in PBS after incubation at 4°C overnight. After washing with PBS/0.05% Tween 20, wells were added with 5-fold serial dilutions of the sera starting at 1/30, then incubated at room temperature for 1 h, washed three times, and added with HRP-conjugated goat anti-mouse IgG1 or IgG2a Abs (Caltag, Burlingame, CA). The reactions were developed by adding 100 μl/well of the TMB Microwell peroxidase substrate (Kierkegaard & Perry, Gaithersburg, MD) and reading at 450 nm after stopping the reaction with 50 μl of 2 M H_2SO_4. The specificity of the binding was assessed by testing these sera against an irrelevant protein. Data are presented as mean values for each Ab isotype. Mouse anti-HER-2/*neu* Ab (Corixa Corp.) was used as a positive control.

Western blot

Tumor lysates were prepared by mechanical disruption in liquid nitrogen, then homogenized and sonicated in RIPA buffer containing 20 mM Tris (pH 8.0), 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM EDTA, 1 mM PMSF, 1× Complete Protease Inhibitors (Roche). Aliquots of the lysates containing 20 μg of protein were boiled and subjected to electrophoresis on 12% denaturing SDS-polyacrylamide. Gels were then transferred to nitrocellulose membranes (Life Technologies, Gaithersburg, MD) and blotted with mouse anti-HER-2/neu Ab (1/10,000; Corixa Corp.) followed by incubation with HRP-conjugated

sheep anti-mouse IgG Ab (1:5000; Amersham Pharmacia Biotech, Piscataway, NJ) and 1 min incubation of the membrane with chemiluminescence (ECL) reagent (PerkinElmer Life Sciences, Boston, MA).

Flow cytometry

Flow cytometry-based analysis was performed as described previously (28). All the procedure was conducted on ice, and washing steps were performed with a PBS-1% sodium azide to avoid internalization/recycling of the receptors. To measure expression of CD4 and/or CD25 molecules on T cells, double staining flow cytometry-based analysis was conducted using FITC-conjugated anti-mouse CD4 and PE-conjugated anti-mouse CD25 (BD PharMingen, San Diego, California). PBL were incubated with Tris-NH_4Cl at room temperature for 5 min to lyse RBC. Cells were then washed twice with PBS-1% sodium azide and blocked by adding the Fc blocker Ab, rat anti-mouse CD16/CD32 (0.3 μg/200 μl/5 $\times$ 10^5 cells; BD PharMingen), while sitting on ice for 20 min, followed by washing twice in cold PBS/1% sodium azide. Cells were then stained with FITC-conjugated anti-CD4 and PE-conjugated anti-CD25 (0.3 μg/200 μl/5 $\times$ 10^5 cells). Cells were finally washed and fixed with 1% ultra pure formaldehyde and read by FACScan within 24 h. Data are presented as the mean fluorescence intensity (MFI), and representative results are also presented as a dot plot.

Statistical analyses

Results were analyzed using Student's *t* test. A value of $p < 0.05$ was considered significant.

Results

HSP110-ICD complex breaks tolerance against rICD by generating Ag-specific T cells

To determine whether the HSP110-ICD complex may break tolerance against rat neu protein in the FVBN202 transgenic mouse, an ELISPOT assay was performed using splenocytes derived from the immunized and naive animals. We evaluated stimulation of ICD-specific IFN-γ- and IL-4-secreting T cells to determine whether HSP110-ICD complex may stimulate $CD8^+$ T cells and/or Th1 cells as well as $CD4^+$ Th2 cells. All animals were ~4 mo of age when sacrificed, and their splenocytes were subjected to ELISPOT assay. All animals revealed similar responsiveness to Con A (Fig. 1, *A* and *B*). Naive and ICD-immunized animals did not show marked IFN-γ or IL-4 secretion upon in vitro stimulation with hICD or rICD. On the other hand, animals immunized with the HSP110-ICD complex elicited marked IFN-γ (Fig. 1*A*) and IL-4 (Fig. 1*B*) secretion upon in vitro stimulation with hICD or rICD. ICD-specific IFN-γ secretion was significantly greater than ICD-specific IL-4 secretion ($p \leq 0.02$). Additionally, ICD-specific IFN-γ secretion was significantly greater ($p = 0.015$) against rICD than that against hICD. There was no IFN-γ or IL-4 response against HSP110 itself. Mice from 6–8 wk of age up to 10 mo of age were evaluated for the pattern of IFN-γ and IL-4 production upon in vitro stimulation with Con A using an ELISPOT assay. No marked difference was detected at different ages, indicating that animals were immunocompetent at the ages tested (data not shown).

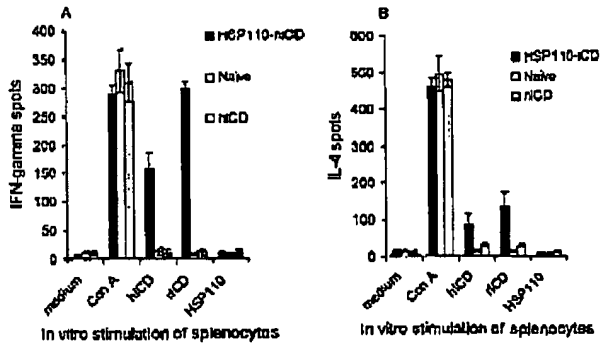

FIGURE 1. The HSP110-ICD complex elicits ICD-specific IFN-γ- and IL-4-producing T cell responses. Six- to 8-wk-old FVBN202 transgenic mice (five mice per group) were immunized i.p. with the HSP110-ICD complex (■) or ICD (□) and received two boosters. The first booster was given after 2 wk, followed by an additional booster 1 mo thereafter. Naive animals remained untreated (□). Animals (~4 mo of age) were sacrificed by cervical dislocation, and their splenocytes were cultured in vitro with Con A, hICD, rICD, or HSP110. Control wells were added with the complete medium. The production of Ag-specific IFN-γ (*A*) or IL-4 (*B*) was evaluated in an ELISPOT assay using a Vision ELISPOT reader (Carl Zeiss).

HSP110-ICD complex protects FVBN202 transgenic mice against spontaneous mammary tumor development

To determine whether the immune responses elicited by HSP110-ICD complex may protect animals from spontaneous mammary tumor development, FVBN202 transgenic mice (eight mice per group) were immunized i.p. with the HSP110-ICD complex (25–50 μg/mouse) at 4–5 mo of age (Fig. 2*A*) or at 2 mo of age (Fig. 2*B*). These times allow comparison of the effectiveness of the vaccine at an earlier stage when atypical hyperplasia of mammary tissues was initiated (2 mo of age) with that at a more progressive stage where infiltrating ductal adenocarcinomas were in place, which histologically resemble human infiltrating ductal mammary adenocarcinoma (22). Animals then received six boosters (the first booster was given 2 wk after priming, and other boosters were given once every month). All nonimmunized animals developed spontaneous mammary tumors by 240–280 days of age when 25% (Fig. 2*A*) or 50% (Fig. 2*B*) of the immunized animals remained tumor free. All animals primed at 4–5 mo of age developed tumor by 320 days of age, when 50% of those primed at 2 mo of age remained tumor free and showed significantly delayed tumor development by 480 days of age. No significant difference was observed between the naive and immunized animals in the former ($p = 0.06$), whereas the difference was significant in the latter ($p = 0.003$).

Both $CD8^+$ and $CD4^+$ T cells are involved in antitumor protection elicited by the HSP110-ICD complex

To determine the T cell subsets involved in the antitumor protection, depletion and challenge experiments were conducted. Six- to 8-wk-old weeks FVBN202 transgenic mice were immunized with the HSP110-ICD complex, followed by two boosters (the first booster was given 2 wk after priming, and second booster was given 1 mo thereafter). Control groups remained nonimmunized. Groups of HSP110-ICD-immunized animals were also depleted of $CD4^+$, $CD8^+$, or $CD4^+/CD8^+$ T cells in vivo in the effector phase of the immune response by i.p. injection of the mAbs, as described in *Materials and Methods*. All animals were then challenged s.c. with a high load (4×10^6 cells/mouse) of the neu-overexpressing MMTC in the right flank and of the neu ANV in the left flank. As indicated in Fig. 3*A*, animals with intact T cells and animals depleted of $CD4^+$ T cells revealed marked protection against tumor growth, whereas naive animals or those depleted of $CD4^+/CD8^+$ T cells developed tumor aggressively. Animals depleted of $CD8^+$ T cells also showed partial protection against the tumor challenge, but it was markedly ($p \leq 0.03$) less than that elicited in the animals with intact T cells or $CD4^+$ depletion. The $CD4^+$-depleted animals revealed stronger protection ($p \leq 0.02$) against the MMTC challenge than the animals with intact T cells. Both naive and HSP110-ICD-immunized animals that were challenged with ANV developed tumors aggressively (Fig. 3*B*).

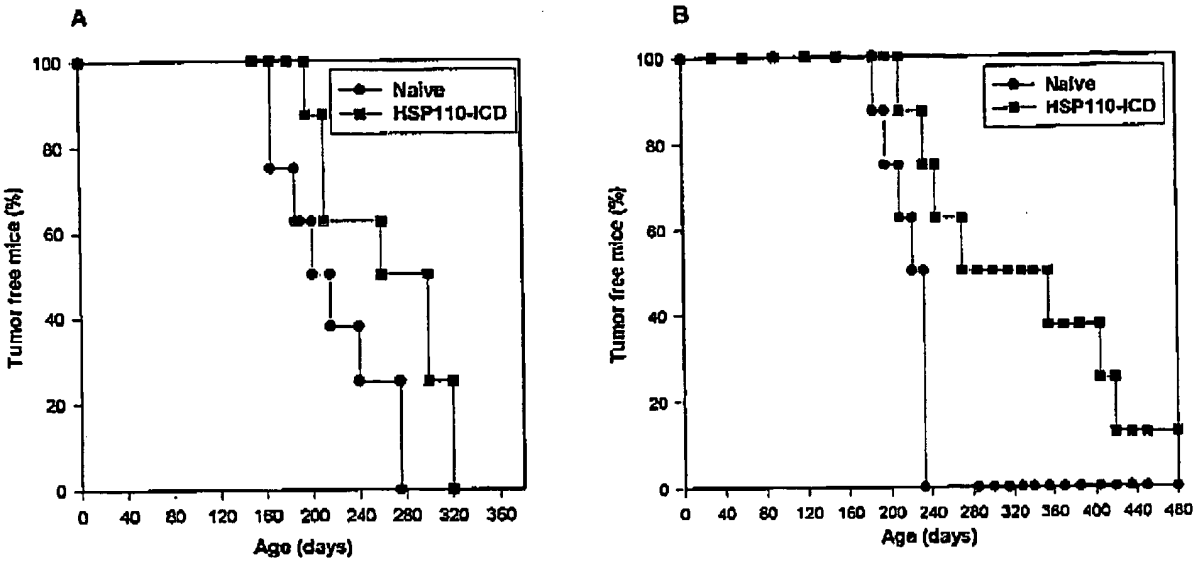

FIGURE 2. The HSP110-ICD complex protects FVBN202 transgenic mice against spontaneous mammary adenocarcinoma. Five- (*A*) or 2-mo-old (*B*) FVBN202 transgenic mice (eight mice per group) were immunized i.p. with the HSP110-ICD complex (■) and boosted six times (the first booster was given after 2 wk, followed by additional boosters once every month). Control groups ($n = 8$) remained nonimmunized (●). Animals were monitored twice weekly for the development of spontaneous mammary tumors. Student's *t* test revealed no significant difference between naive and immunized animals in the former (*A*; $p = 0.06$), whereas the difference was significant in the latter (*B*; $p = 0.003$).

Antitumor protection elicited by the HSP110-ICD complex is correlated with Th1-type-dependent IgG2a Ab response

Since IFN-γ was the major cytokine generated upon immunization with the HSP110-ICD complex, and $CD4^+$ T cells revealed a partial effect against the tumor in the challenge studies, we wondered whether only a subtype of $CD4^+$ T cells, Th1 cells, may be protective. To address this question, IgG1 (indicative of Th2 activation) and IgG2a (indicative of Th1 activation) Ab responses were evaluated in the sera of HSP110-ICD-immunized animals that either eventually developed tumors or remained tumor free. Naive (tumor-free or tumor-bearing) animals were also used as controls. As indicated in Fig. 4, naive animals revealed no Ab responses against rICD, whereas animals immunized with the HSP110-ICD complex showed IgG1 (*A*) and IgG2a (*B*) Ab responses against rICD. Both tumor-free and tumor-bearing animals showed similar IgG1 Ab responses (*A*), whereas the IgG2a Ab response (*B*) was markedly higher in the tumor-free animals than in the tumor-bearing animals ($p = 0.002$). Mouse anti-HER-2/*neu* Ab was used as a positive control. No Ab was detected against HSP110 itself (data not shown).

Later development of spontaneous mammary tumors in HSP110-ICD-immunized animals is not due to neu protein loss

To determine whether later development of spontaneous mammary tumors in some of the immunized animals might be due to neu Ag loss under immune pressure, Western blot analysis was performed on mammary tumors obtained from tumor-bearing animals. Naive mice and animals immunized with HSP110-ICD were compared. As indicated in Fig. 5, HSP110-ICD immunization did not result in neu Ag loss. Similar expression of neu was detected in the mammary tumors of naive and immunized animals.

Development of spontaneous mammary tumors coincides with elevated numbers of $CD4^+CD25^+$ regulatory T cells

To determine whether suppressor T cells may prevent complete eradication of mammary tumors following immunization, PBL from tumor-free and tumor-bearing animals were subjected to flow cytometry-based analyses. Both naive animals (5–7 mo of age; Figs. 6, *A–C*) and animals immunized with the HSP110-ICD complex (5–12 mo of age; Fig. 6, *D–F*) were evaluated. The frequency of $CD4^+CD25^+$ regulatory T cells was determined using FITC-conjugated anti-CD4 and PE-conjugated anti-CD25 mAbs. Naive or HSP110-ICD-immunized animals that developed spontaneous mammary tumors, regardless of their age, revealed markedly higher $CD4^+CD25^+$ T cells than tumor-free animals. Results are presented as the MFI (Fig. 6, *A* and *D*; $n = 4$), and representative results are also presented as a dot plot (Fig. 6, *B*, *C*, *E*, and *F*).

Discussion

In the present study we used the chaperoning properties of HSP110 to generate a noncovalent binding complex with the intracellular domain of human HER-2/*neu*. This HSP110-ICD complex was then evaluated for its antitumor potential in an FVBN202 transgenic mouse that overexpresses rat *neu* oncogene and develops spontaneous mammary tumors with many features similar to human breast cancer. These transgenic mice tolerate the neu protein and there is no pre-existing immunity against the neu protein. Therefore, breaking tolerance is very difficult in the FVBN202 transgenic mouse. We used hICD since it is 92% identical in amino acid sequence to rICD, and this Ag mimicry has been previously shown to break tolerance against rat neu protein (23). The antitumor ability of the HSP110-ICD complex was evaluated in both therapeutic and prophylactic studies. T cell subsets involved in antitumor protection were identified and the mechanisms preventing the vaccine from completely eradicating mouse mammary tumors were evaluated.

We have previously shown that the HSP110-ICD complex could elicit ICD-specific immune responses in A2/Kb transgenic mice in which both $CD8^+$ and $CD4^+$ T cells were involved in ICD-specific IFN-γ secretion, as determined by depletion of $CD4^+$ and/or $CD8^+$ T cells in vivo (16). Using irrelevant protein indicated that $CD8^+$ T cells were specific for ICD (16). In the present study we

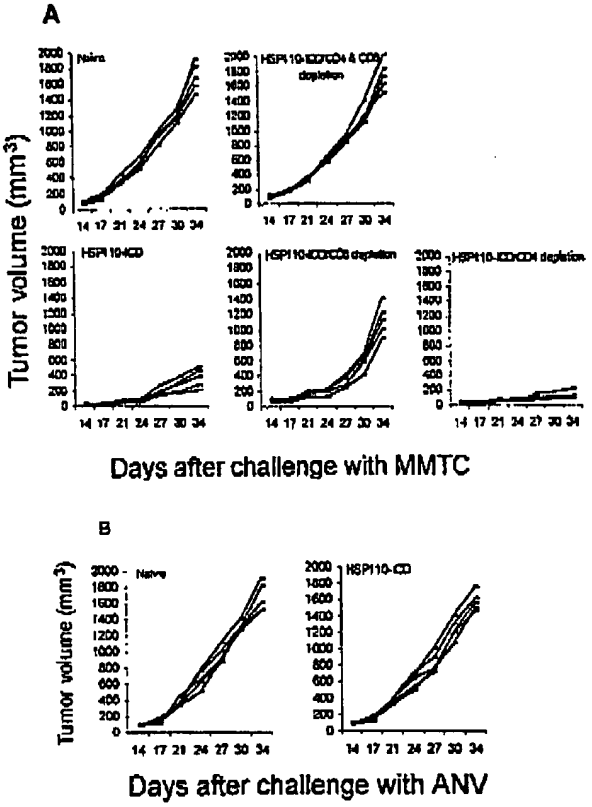

FIGURE 3. Both $CD8^+$ and $CD4^+$ T cells are involved in antitumor protection elicited by HSP110-ICD immunization. Six- to 8-wk-old FVBN202 transgenic mice (five mice per group) were immunized i.p. with the HSP110-ICD complex and received two boosters. The first booster was given after 2 wk, followed by an additional booster 1 mo thereafter. Naive animals remained untreated. Groups of HSP110-ICD-immunized animals were also depleted of $CD4^+$, $CD8^+$, or $CD4^+/CD8^+$ T cells by i.p. injection of mAbs GK1.5 and/or 2.43 before tumor challenge. Animals were then challenged with MMTC in the right flank (*A*) and ANV in the left flank (*B*). Animals were monitored weekly for the development of mammary tumors. Since there was no protection against challenge with ANV, the results from naive and HSP110-ICD immunized animals (no T cell depletion) are presented (*B*).

demonstrated that the HSP110-ICD complex was capable of breaking tolerance against rat neu protein, as indicated by inhibition of spontaneous mammary tumor development as well as generation of rICD-specific IFN-γ- and IL-4-producing T cells. This is very important, since unlike the human protein, there is no preexisting immunity against the rat neu protein in this transgenic model. Immunization of animals with ICD alone did not result in Ag-specific immune responses. Although there was no significant difference between IFN-γ and IL-4 production upon Con A stimulation in vitro, the HSP110-ICD complex induced significantly greater IFN-γ than IL-4 production ($p = 0.02$). This may indicate that IFN-γ-producing T cells are more important than IL-4-producing T cells in anti-tumor immune responses. Similar results were reported by others, showing that IFN-γ plays a central role in protection against mouse mammary tumors (29–31). They reported that induced IFN-γ can affect the behavior of neu-overexpressing tumor cells by inhibiting their angiogenic phenotype and their proliferation, and down-regulating their membrane expression of HER-2/*neu* (31). In addition, there was no marked difference in IL-4 production against hICD or rICD, whereas ICD-specific IFN-γ production was significantly higher ($p = 0.01$) against rICD than against hICD. This may suggest that once tolerance is broken by HSP110-ICD immunization, overexpression of rat neu protein in vivo (initiated at puberty) may function as an internal continuous booster, leading to selective expansion of T cells bearing receptors with the highest affinity for rat neu protein and functional avidity maturation of Ag-specific T cells (32). We used mouse HSP110 in the vaccine complex, and no IFN-γ or IL-4 secretion was detected against self HSP110. This is very important, since breaking tolerance against self HSP110 could lead to an autoimmune reaction.

The ICD-specific immune response elicited by the HSP110-ICD complex was effective against spontaneous mammary tumors in both a therapeutic and a prophylactic setting. In therapeutic studies, initiation of immunization at an earlier stage, i.e., 2 mo of age (where atypical hyperplasia of mammary tumors was initiated) revealed stronger tumor inhibition than at a later stage (when infiltrating ductal adenocarcinomas were in place). This indicates that the HSP110-ICD complex was more effective at earlier stages than at the progressive stage of mammary carcinomas. Others have also indicated that antitumor protection was evident when immunization started at 2–3 mo of age, but not at 6 mo of age (22). Depletion studies at the effector phase of the immune response revealed that both $CD8^+$ and $CD4^+$ T cells were involved in the protective effect obtained against the mammary tumor. These T cell responses were Ag specific, since both naive and HSP110-ICD-immunized animals revealed no antitumor protection against challenge with the ANV of MMTC. Animals depleted of $CD4^+/CD8^+$ T cells, but with intact NK cells, revealed no protection against challenge with MMTC. This indicates that $CD4^+$ and/or $CD8^+$ T cells were involved in antitumor protection whereas the presence of NK cells did not protect animals against tumor development. $CD8^+$-depleted animals revealed less protection upon tumor challenge than animals with intact T cells or those depleted of $CD4^+$ T cells. Interestingly, animals depleted of $CD4^+$ T cells revealed the strongest protection against tumor challenge even compared with those with intact T cells. Together, these results suggest that $CD8^+$ T cells are more important than $CD4^+$ T cells in antitumor immune responses against mouse mammary tumor. We performed depletion studies in a prophylactic tumor setting, since it is not feasible to deplete $CD4^+$ and/or $CD8^+$ T cells in vivo in a spontaneous tumor setting due to a longer experimental period (>1 year). Therefore, results obtained from depletion studies may not be directly applicable to the spontaneous tumor setting. However, the present findings are consistent with our previous studies, which demonstrated that both $CD4^+$ and $CD8^+$ T cells were involved in ICD-specific immune responses (16). In the present studies while $CD8^+$ T cells fully protect animals against tumor challenge, partial protection by $CD4^+$ T cells may indicate that the latter have a double function, for and against tumor challenge. IL-4-secreting Th2 cells may favor tumor growth, and IFN-γ-secreting Th1 cells may have antitumor function. This may explain the increased IgG2a Ab (indicative of Th1 response) titers in the sera of tumor-free animals, whereas tumor-bearing animals revealed decreased IgG2a Ab titers. Since ICD is an intracellular protein and Abs could not access this Ag within the cell, IgG2a Ab may be more indicative of Th1-type protection than direct Ab protection. This finding is consistent with other reports indicating that elevated IgG2a Ab is correlated with antitumor protection in FVBN202 transgenic mice (31). Additionally, Th1 cells are responsible for the secretion of IFN-γ, which was the major cytokine secreted in an ICD-specific fashion. No neu-specific Ab response was detected

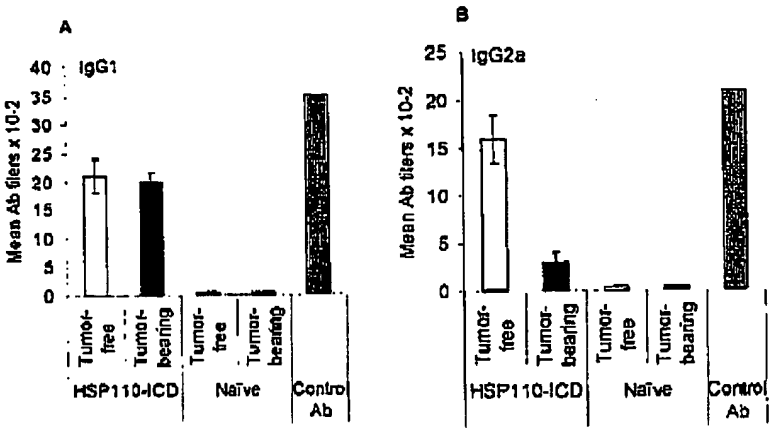

FIGURE 4. Antitumor protection elicited by the HSP110-ICD complex is correlated with Th1-type-dependent IgG2a Ab response. Two-month-old FVBN202 transgenic animals (four mice per group) were immunized with the HSP110-ICD complex and boosted three times (the first booster was given after 2 wk, followed by additional boosters once every month). Control groups remained nonimmunized. Sera were collected from tumor-free naive animals (3–4 mo of age), tumor-bearing naive animals (6–8 mo of age), and tumor-free or tumor-bearing HSP110-ICD immunized animals (9–12 mo of age). Sera were subjected to ELISA to determine rICD- or HSP110-specific IgG1 (*A*) and IgG2a (*B*) Ab responses. No Ab was detected against HSP110 (data not shown). Results are presented as mean Ab titers. Mouse anti-HER-2/neu Ab was used as a positive control.

In naive animals regardless of whether they were carrying the tumor. Again, this indicates that FVBN202 transgenic mice tolerate the tumor. We have previously shown that HSP110 in a complex with ICD could generate ICD-specific Ab responses in A2/Kb transgenic mice. Others also reported that gp96 isolated from a syngeneic cell line transduced with BHV-1 glycoprotein D induced glycoprotein D-specific Abs (33). However, we used recombinant HSP110 rather than cell-derived HSP110, and then loaded it with a large Ag, ICD, which comprises numerous epitopes, including Th epitopes as well as B cell defined epitopes. Therefore, the induction of Ab responses in our system using large protein as an Ag is easier than that using cell-derived HSPs that may or may not carry Th or B cell defined epitopes.

Why does immunotherapy in general and HSP110-ICD complex in particular fail to completely eradicate mammary tumors? Is it because of tumor escape or failure of the immune system? We explored whether FVBN202 transgenic mice immunized with the HSP110-ICD complex that eventually developed spontaneous mammary tumors (with a marked delay) might lose neu expression due to immune pressure and thereby escape antitumor immune responses. Using Western blot analysis of the tumors, we detected similar levels of neu protein expression in tumor-bearing naive and immunized animals. We also conducted RT-PCR analyses of RNA isolated from mammary tumors and did not detect neu loss at the transcription level (data not shown). These data indicated that later development of spontaneous mammary tumors was not due to neu Ag loss. Since we detected ICD-specific IL-4 secretion in HSP110-ICD-immunized animals and depletion of $CD4^+$ T cells resulted in better protection against challenge with MMTC, we explored whether a subset of $CD4^+$ T cells, Th2 cells, might be involved in determining the outcome of HSP110-ICD immunotherapy. It was previously shown that breast cancer patients had elevated numbers of $CD4^+CD25^+$ regulatory T cells (Th2 cells) compared with healthy individuals (26). Therefore, we evaluated the $CD4^+$ $CD25^+$ T cell population in both tumor-free and tumor-bearing animals to determine whether these suppressor T cells may be elevated in tumor-bearing animals. Interestingly, we detected elevated numbers of $CD4^+CD25^+$ regulatory T cells in PBL of most tumor-bearing animals, but not in PBL of tumor-free animals. The presence of these regulatory T cells may account for the secretion of IL-4, as detected by ELISPOT assay. It may also explain the stronger antitumor protection in $CD4^+$-depleted animals than in $CD8^+$-depleted animals. Depletion of $CD4^+$ T cells would deplete Th1 and Th2 cells where $CD8^+$ T cells remain intact and may function better against the mammary tumor in the absence of Th2 regulatory T cells. On the other hand, the presence of Th2 cells would suppress the antitumor function of both $CD8^+$ T cells and Th1 cells, leading to the development of tumor at a later time. It was reported that the prevalence of $CD4^+CD25^+$ regulatory T cells was significantly higher in the PBL of breast cancer patients compared with normal donors (26). We also observed that immunization of animals after surgical removal of aggressively growing mammary tumors failed to protect animals against tumor relapse (data not shown). This may indicate that initiation of immunization in the presence of elevated $CD4^+CD25^+$ regulatory T cells would fail to inhibit tumor development. It was previously shown that repetitive antigenic stimulation of T cells could generate $CD4^+$ regulatory T cells (34); therefore, neu overexpression in vivo may function as repetitive stimulation for the generation of regulatory T cells. This hypothesis remains to be investigated. $CD4^+CD25^+$ regulatory T cells are engaged in the maintenance of immunogenic self-tolerance by actively suppressing the activation and expansion of self-reactive T lymphocytes that may cause autoimmune disease. The majority of these regulatory T cells constitutively express CD25. To date, numerous approaches have been used in the immunotherapy of cancers; however, little success has been achieved. This failure has been shown in several cases to be associated with the presence of $CD4^+CD25^+$ regulatory T cells (35–37). These regulatory T cells constitutively express CTLA-4 and suppress Ag-specific and polyclonal activation and proliferation of other T cells, including CTLA-4-deficient T cells (38, 39). These regulatory T cells also suppress the antitumor function of effector $CD4^+$ (Th1) and $CD8^+$ T cells, thereby preventing complete eradication of tumors (35, 36, 40). Therefore, this necessitates designing strategies to overcome these regulatory T cells to improve active specific immunotherapy of cancers. The Ags that may selectively up-regulate such suppressive T cells need to be identified.

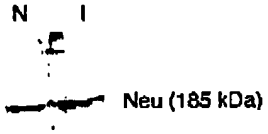

FIGURE 5. HSP110-ICD immunization does not result in neu Ag loss. Seven- to 10-mo-old tumor-bearing FVBN202 transgenic mice (naive or HSP110-ICD immunized) were sacrificed, mammary tumors were removed from both naive (N) and immunized (I) mice, tumor lysates were prepared, and the expression of the neu protein was examined by Western blot analysis using mouse anti-HER-2/neu Ab.

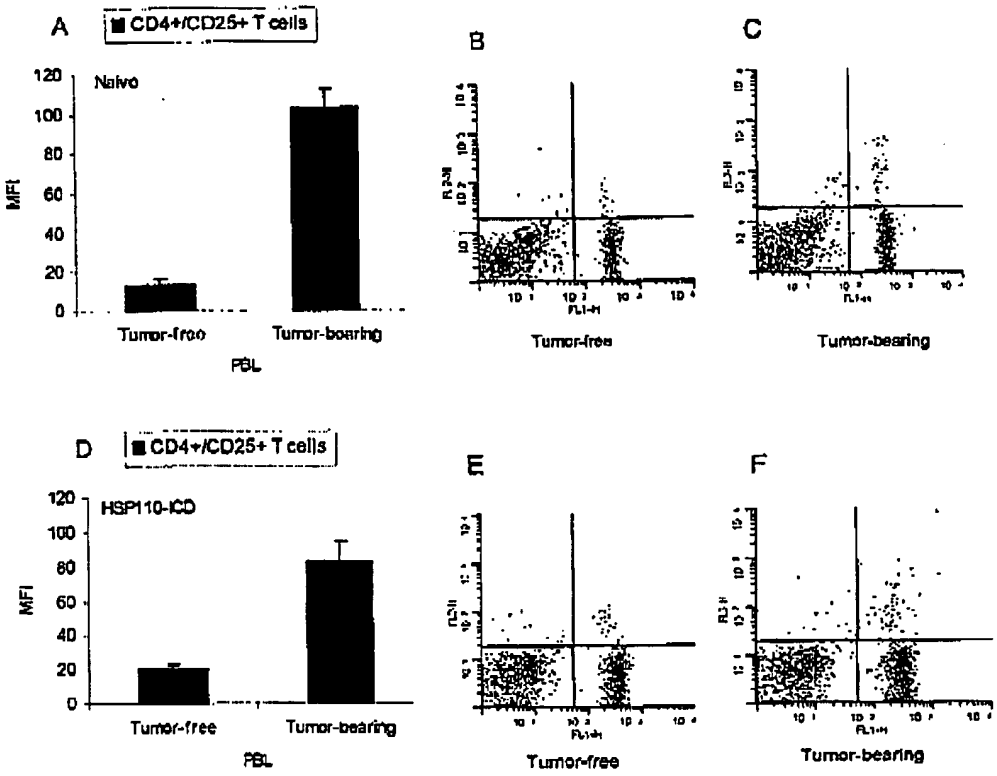

FIGURE 6. Development of spontaneous mammary tumors in the presence of elevated $CD4^+$ $CD25^+$ regulatory T cells. Naive tumor-free or tumor-bearing FVBN202 transgenic mice (*A–C*) and animals immunized with the HSP110-ICD complex and remaining tumor free or eventually developing tumors (*D–F*) were evaluated for the presence of $CD4^+$ $CD25^+$ regulatory T cells in their PBL using PE-conjugated anti-CD25 and FITC-conjugated anti-CD4 Abs in a flow cytometry-based analyses. Results are presented as the MFI ($n = 4$). Representative results are also presented as a dot plot (*B* and *C*, *E* and *F*) to demonstrate that the MFI reflects the number of suppressor T cells.

The findings described here support the use of HSP110-ICD vaccination in the treatment of patients with cancers overexpressing HER-2/*neu*. These findings also suggest that IFN-γ-secreting T cells are protective against mouse mammary tumors, whereas IL-4-secreting T cells may suppress antitumor immune responses. Up-regulation of $CD4^+$ $CD25^+$ regulatory T cells in tumor-bearing animals suggests that these immunotherapeutic approaches may need to include strategies to overcome the immunosuppressive function of these regulatory T cells. There are other suppressor T cells, such as $CD8^+$ T cells and γδ T cells, that need to be investigated to determine their roles in the tumor-immune system interaction. Finally, the FVBN202 transgenic model examined here represents a "high bar" for evaluation of the vaccine. These mice are under continuous genetic pressure for tumor development. In this respect, the results described here demonstrate a significant antitumor activity of the HSP110-ICD complex.

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